Hox gene *Ultrabithorax* regulates distinct sets of target genes at successive stages of *Drosophila* haltere morphogenesis

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Hox genes encode highly conserved transcription factors that regionalize the animal body axis by controlling complex developmental processes. Although they are known to operate in multiple cell types and at different stages, we are still missing the batteries of genes targeted by any one Hox gene over the course of a single developmental process to achieve a particular cell and organ morphology. The transformation of wings into halteres by the Hox gene *Ultrabithorax* (*Ubx*) in *Drosophila melanogaster* presents an excellent model system to study the Hox control of transcriptional networks during successive stages of appendage morphogenesis and cell differentiation. We have used an inducible misexpression system to switch on *Ubx* in the wing epithelium at successive stages during metamorphosis—in the larva, prepupa, and pupa. We have then used extensive microarray expression profiling and quantitative RT-PCR to identify the primary transcriptional responses to Ubx. We find that Ubx targets range from regulatory genes like transcription factors and signaling components to terminal differentiation genes affecting a broad repertoire of cell behaviors and metabolic reactions. Ubx up- and down-regulates hundreds of downstream genes at each stage, mostly in a subtle manner. Strikingly, our analysis reveals that Ubx target genes are largely distinct at different stages of appendage morphogenesis, suggesting extensive interactions between Hox genes and hormone-controlled regulatory networks to orchestrate complex genetic programs during metamorphosis.

appendage specialization | homeotic genes | serial homology

Studies of Hox genes first prompted the realization that bilaterians share an extensive genetic makeup for body patterning (1) and have since spearheaded the quest for the genetic changes that drive morphological evolution (2). Hox genes encode homeodomain transcription factors that operate in many tissues and cell types and modulate a wide range of cell responses by controlling expression of subordinate target genes. Despite their highly conserved roles in body regionalization, only a limited number of Hox-controlled morphogenetic and differentiation programs have been studied in detail. For all of these processes, we do not understand the batteries of genes modulated by Hox proteins to bring about complex developmental transformations.

The systematic elucidation of Hox targets has been hampered by the complexity and multiplicity of the regulatory networks that Hox genes control and the short and degenerate binding sites of Hox proteins. It has been further complicated by the functional redundancy of the Hox proteins and the cross-regulatory interactions between them. These factors have made it difficult to elucidate the complete set of target genes modulated by any one Hox gene over the course of a single developmental process (3–8). Thus, it remains an open question of how these widely conserved patterning genes link to the actual effectors of cell and organ morphology at a genome-wide level over time.

The dorsal flight appendages of *Drosophila melanogaster* present an excellent system to study Hox-controlled morphogenesis. *Drosophila* and flies in general have evolved from four-winged ancestors by modifying their hind wings into drumstick-like balancing organs called halteres (9). *Ultrabithorax* (*Ubx*) is expressed in the hind wings of all winged insects studied (10–13), but it is only in Dipterans that *Ubx* brings about the striking morphological transformation of hind wings into halteres. Compared with the wing blade, the haltere capitellum is reduced in size by a fivefold reduction in cell number and an eightfold reduction in apical cell surface area (14, 15). It forms a balloon shape rather than a flat bilayer because of changes in cell adhesion, it shows no differentiation between vein and intervein cells, and it lacks marginal bristle rows (15).

The wing epithelium develops in the absence of any Hox input (16) and represents one of the best-studied model systems in terms of genetic networks underlying pattern formation, growth, and differentiation. *Ubx* modifies this wing developmental network by regulating the expression of target genes to generate halteres (17, 18).

Molecular genetic studies have shown that many key patterning genes in the larval wing primordium are indeed regulated by Ubx, directly or indirectly, in the haltere primordium (14, 17, 19–25). This finding has prompted the use of microarray expression profiling to systematically identify differentially expressed genes between wing and haltere imaginal discs (26, 27). These genome-wide approaches, however, were not designed to reveal the primary transcriptional responses to Ubx and have uncovered the cumulative effects from *Ubx* function throughout development. Moreover, most studies have focused disproportionately on the role of *Ubx* in appendage patterning and growth in larval stages, whereas the actual transformation in cell and organ morphology elicited by *Ubx* occurs later during metamorphosis (15).

Elucidation of the architecture of Hox-controlled gene regulatory networks is fundamental to understanding morphogenesis and cell differentiation and how these processes underlie diversification of serially homologous structures like wings and halteres. To identify the genes directly regulated by Ubx to distinguish halteres from wings, we used the TARGET version of the GAL4/*UAS* system (28) coupled with microarrays to profile transcriptional changes in wings shortly after *Ubx* misexpression (unlike previous studies that compared wings with halteres that express *Ubx* throughout development). Altering the temperature, we were able to switch on ectopic *Ubx* specifically in developing wing blades at levels similar to those observed in normal halteres and measure the immediate transcriptional responses to *Ubx* at successive stages during larval, prepupal, and pupal development.

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Results

Genetic System for Precisely Controlled *Ubx* Misexpression in Developing *Drosophila* Wings. We generated an experimental fly line that produces a complete transformation of the wing blade to haltere capitellum (Fig. 1 *A* and *D*). This line combined the *nab-Gal4*^{NP3537} driver, a *tub-GAL80*^{ts} transgene, and a *UAS-UbxIa* transgene (or a *UAS-eGFP* control), and it satisfied a number of essential criteria to study Hox gene function on a genome-wide scale.

With this system, *Ubx* (or *eGFP*) was misexpressed specifically in the developing wing blade, a tissue of low complexity composed primarily of the vein and intervein cells, the marginal bristle cells, and a few sensilla (Fig. 1 *C*, *F*, and *G*). By measuring gene expression in the fairly homogeneous wing epithelium, we ensured the maximum sensitivity to detect transcriptional responses, because each cell type made a relatively big contribution to the total transcript population.

The experimental line used did not interfere with normal wing development at 19 °C; a homeotic transformation of wings into halteres was only observed after a temperature shift (ATS) from 19 °C to 29 °C. The transformation was evident both at the organ (Figs. 1 *B* and *E* and 2 *A*–*C*) and cell level (Fig. 2 *A*′–*C*′). The wing blade turned into a hollow balloon composed of fewer cells that resembled haltere cells. Normal wing cells are squamous stellate polygons that project one long hair. After misexpressing Ubx, wing cells were transformed into haltere-like cuboidal cells that have a smaller rectangular surface area and produce multiple short hairs.

We quantified the levels of ectopic Ubx protein in the wing epithelium of the experimental line ATS from 19 °C to 29 °C (Fig. 3 *A*–*J*). This quantification was essential, because the activity of Hox proteins is concentration-dependent (15, 29). We collected

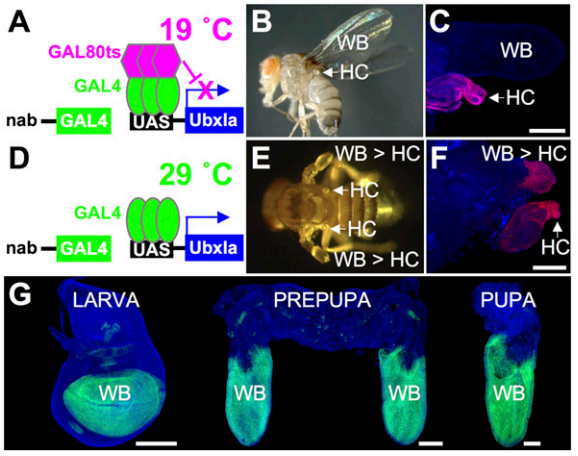

Fig. 1. Controlled *Ubx* misexpression in the developing wing epithelium. (*A*) The GAL4 activator is expressed from the *nab* locus (*nab*^{NP3537}) and binds its cognate *UAS* sequences controlling expression of the *UbxIa* transgene. At 19 °C, the temperature-sensitive repressor GAL80ts is functional and inhibits GAL4-mediated activation and *UbxIa* expression. (*B*) Flies grown at 19 °C develop a normal wing blade (WB) that is markedly different from the haltere capitellum (HC; arrow). (*C*) The wing develops in the absence of any Hox input (nuclei stained in blue), whereas Ubx protein (magenta) is detected in the haltere. (*D*) After temperature shift from 19 °C to 29 °C, the GAL80ts repressor is inactivated, and the GAL4 activator drives expression of *UbxIa*. (*E*) Almost complete homeotic transformation of the adult WB into HC (WB > HC). Normal HC are marked with arrows. Other parts of the notum remain unaffected. (*F*) In prepupal thoraces of these flies, Ubx protein (magenta) is detected not only in the haltere but also in the transformed WB. (*G*) The WB is an epithelial bilayer that forms from the central pouch of the wing disc epithelium. Using the TARGET system, eGFP (fixed protein in green) or Ubx is switched on specifically in the wing blade (nuclei stained in blue) at three successive stages: late third instar larval stage (*Left*), prepupal stage (*Center*), and early pupal stage (*Right*). (Scale bar, 100 μm.)

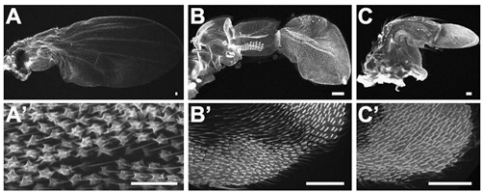

Fig. 2. Effects of *Ubx* misexpression in the wing epithelium. (*A*, *A*′, *B*, *B*′, *C*, and *C*′) Late pupal phalloidin (F-actin) staining of (*A*) WT wing, (*B*) WT haltere, and (*C*) experimental wing with a complete homeotic transformation of wing blade into haltere capitellum. High magnification of the same appendages showing the characteristics of (*A*′) wing cells, (*B*′) haltere cells, and (*C*′) transformed wing cells that have acquired the properties of haltere cells. (Scale bar, 25 μm.)

our microarray samples 16 h ATS when ectopic Ubx levels in the wing blade reached about 90% of the levels normally observed in the homologous region of the haltere (Fig. 3 *F* and *J*). This is about 10 h after the first detection of ectopic Ubx protein and about 5 h after ectopic Ubx levels reached 50% of WT haltere levels (Fig. 3*I*). Thus, we presumed that assaying gene expression 16 h ATS would preferentially identify primary rather than secondary transcriptional responses to Ubx. This assumption was confirmed by all our subsequent analyses.

We confirmed by in situ hybridization that ectopic Ubx in the wing was capable of regulating two enhancers controlling the *spalt* and *knot* genes targeted by Ubx in WT halteres (19, 21). This analysis also suggested that samples collected 16 h ATS were appropriate to capture the primary transcriptional responses to Ubx and that, in some cases (exemplified by *kn*$^{Mel701–1991}$), they captured a partial and not full response of primary target genes to Ubx (Fig. 3 *K*, *K*′, *K*″, *L*, *L*′, and *L*″).

Experimental Design. Our core Affymetrix array analysis involved comparison of the transcriptional profile of experimental *UAS-Ubx* wings with that of control *UAS-eGFP* wings (Fig. 4 *A* and *C*).

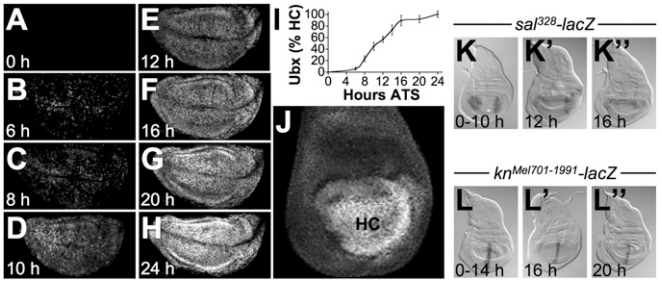

Fig. 3. Quantification of ectopic Ubx levels in the wing epithelium and regulation of targeted enhancers. (*A*–*J*) Immunofluorescent detection of ectopic Ubx in experimental late third instar larval wing discs collected at different time points after temperature shift (ATS), and comparison with Ubx levels in the homologous region in the WT haltere (HC, haltere capitellum). (*A*) Disc grown at 19 °C shows no Ubx expression; (*B*) disc collected 6 h ATS with 6% Ubx, (*C*) disc collected 8 h ATS with 23% Ubx, (*D*) disc collected 10 h ATS with 45% Ubx, (*E*) disc collected 12 h ATS with 57% Ubx, (*F*) disc collected 16 h ATS with 89% Ubx, (*G*) disc collected 20 h ATS with 92% Ubx, and (*H*) disc collected 24 h ATS with 101% Ubx. (*I*) Plot summarizing ectopic Ubx protein levels in wing blades expressed as a percentage of normal levels in WT HC. Error bars denote the SD from the mean of calculated ratios. (*J*) Normal Ubx expression in WT late third instar larval haltere disc. Ubx is expressed at higher levels in the central pouch region, which will give rise to HC. (*K*, *K*′, *K*″, *L*, *L*′, and *L*″) Third instar larval wing disc in situ hybridizations for *lacZ* reporter transcripts controlled by the *sal*^{328} and *kn*$^{Mel701–1991}$ enhancers at different time points ATS. (*K*) Normal *sal*^{328} pattern detected up to 10 h ATS. (*K*′) A substantial reduction in *sal*^{328}*-lacZ* levels is detected at 12 h, and (*K*″) complete repression is evident 16 h ATS. (*L*) Normal *kn*$^{Mel701–1991}$ pattern detected up to 14 h ATS. A partial reduction in *kn*$^{Mel701–1991}$*-lacZ* levels is detected (*L*′) 16 h or even (*L*″) 20 h ATS.

Pair-wise comparisons were carried out at three successive developmental stages that span the critical period of appendage morphogenesis and cell differentiation: (*i*) late larval stage, (*ii*) prepupal stage shortly after disc eversion, and (*iii*) early pupal stage at the onset of cell differentiation (Fig. S1).

Most importantly, pair-wise comparisons were carried out with samples collected at 29 °C 16 h ATS (TARGET system on) as well as with samples developed exclusively at 19 °C (TARGET system off) (Fig. 4 *A* and *C*). This allowed us to distinguish the *Ubx*-dependent effects from the intrinsic expression differences between the fly lines used and the temperature-induced responses. Hierarchical clustering of all 48 samples showed that the developmental stage had the largest influence on the overall expression profile followed by the temperature, which had a greater effect than the misexpressed transgene (Fig. S2). Overall, standard quality controls showed that we generated a very precise dataset (Figs. S3 and S4).

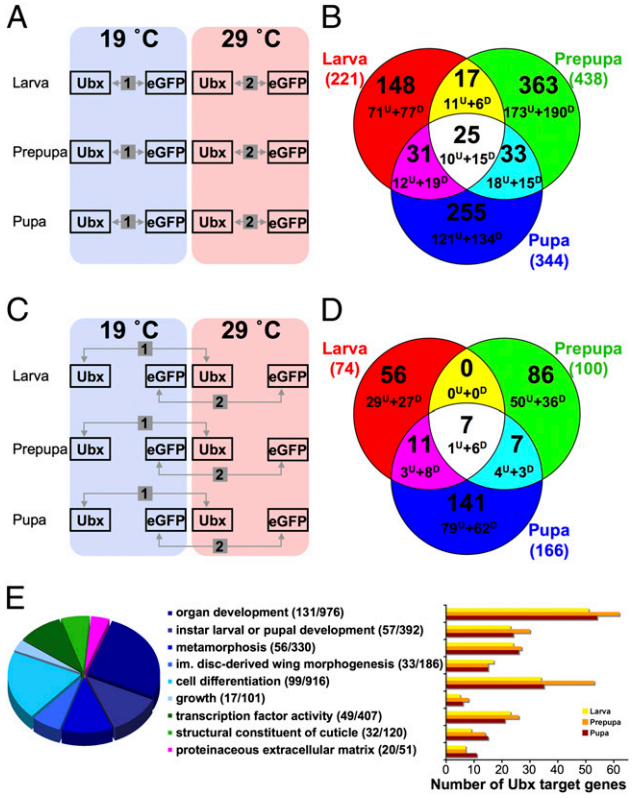

Fig. 4. Analysis of microarray data. (*A*) Primary analysis for identification of Ubx targets. Pair-wise comparisons were carried out at each developmental stage between experimental *UAS-Ubx* and control *UAS-eGFP* samples, both at nonexpressing (19 °C) and expressing (29 °C) conditions (double arrows labeled 1 and 2). Genes differentially expressed at 29 °C but not 19 °C were considered as the putative direct Ubx targets for the particular developmental stage and were then compared with the other two stages. (*B*) Venn diagram showing the number of putative Ubx targets identified by the primary analysis at the three developmental stages. Genes up-regulated by Ubx are labeled with ^{U}, and genes down-regulated are labeled with ^{D}. (*C*) Second analysis for the identification of Ubx targets. For each developmental stage, pair-wise comparisons were carried out between the 19 °C and 29 °C *UAS-Ubx* samples as well as between the 19 °C and 29 °C *UAS-eGFP* samples (double arrows labeled 1 and 2). The genes that were identified in the primary analysis and changed their expression between temperatures only in the presence of Ubx were scored as targets at the particular developmental stage and were then compared with the other two stages. (*D*) Venn diagram showing the number of putative Ubx targets identified by the second analysis as in *B*. (*E*) Gene ontology analysis of Ubx targets identified by the primary analysis. Overrepresented GO annotations are shown color-coded in the left pie chart, with biological processes in shades of blue, molecular functions in shades of green, and a cellular component in magenta. Each GO term is followed in parentheses by the number of associated genes in the entire set of Ubx targets and in the *Drosophila* genome. All terms are significant at the 0.05 level except growth, with an adjusted *P* value of 0.17. The bar chart shows the number of Ubx target genes annotated with each GO term in each developmental stage.

Ubx Regulates Hundreds of Target Genes in a Stage-Specific Manner. For each developmental stage, the primary analysis of the microarray data identified as putative direct Ubx targets those genes that were expressed differentially between the experimental *UAS-Ubx* and control *UAS-eGFP* samples at 29 °C but not 19 °C (Fig. 4*A*). This analysis controlled well for the intrinsic expression differences between the nonisogenic experimental and control lines, regardless of *Ubx* expression. The sets of target genes were then compared between stages to study how transcriptional responses to Ubx change during development. At a false discovery rate (FDR) threshold of 5%, this primary analysis showed that Ubx regulates 872 downstream protein-coding genes listed in Dataset S1. The large majority of these targets (766 genes; 88%) responded to Ubx at a single developmental stage, and only 106 genes (12%) were regulated at two or all three of the stages examined (Fig. 4*B*).

Ubx seems to function both as an activator and repressor, because there was no significant difference in the number of identified up- and down-regulated genes (Fig. 4*B*). The number of genes identified as Ubx targets at the larval stage was smaller than that recovered in later stages. This difference is most likely an artifact. Expression profiling at the larval stage was carried out with entire wing discs, a more complex tissue than the dissected wing blades homogeneously expressing *Ubx* (or *eGFP*) used at prepupal and pupal stages (Fig. 1*G* and Fig. S1). This may have resulted in lower sensitivity at the larval stage.

We carried out an additional, more stringent analysis to eliminate target genes that might respond to the temperature shift differentially in experimental and control samples, independently of *Ubx* expression (Fig. 4*C*). For each developmental stage, genes scored as direct Ubx targets in the primary analysis were required to change their expression after temperature shift only in the presence of *Ubx* but not *eGFP* (at 10% FDR). This more stringent analysis reduced the identified set of putative targets to about one-third (308 genes) (Dataset S1) but confirmed the finding that Ubx regulates the majority of genes in a stage-specific manner (Fig. 4*D*). The majority of the target genes eliminated by this more stringent analysis were most likely false-negatives, excluded because of the relatively large effects of temperature on gene expression (Fig. 5*C* and Fig. S2).

Ubx Targets Encompass Diverse Developmental Roles. We searched for overrepresented gene ontology (GO) annotations describing the biological roles of *Ubx* targets identified in the primary analysis (Fig. 4*E* and Dataset S2). Top-scoring GO terms describe developmental processes and stages pertaining to our study, including organ/appendage development, metamorphosis, wing morphogenesis, growth, cell differentiation, and larval/pupal development. Stage-specific analyses produced results similar to the global analysis on all identified targets as well as GO terms particular to each stage (Dataset S2).

In accordance with previous studies of Hox genes in diverse developmental contexts, Ubx controls the expression of several genes in the haltere with transcriptional regulatory activity as well as genes involved in programmed cell death (Fig. 6*I* and Dataset S1). Our study also identified categories of terminal differentiation genes that did not feature prominently in previous studies for Ubx targets in the haltere. In particular, the GO analysis highlighted many genes encoding components of the cuticle and extracellular matrix (Figs. 4*E* and 6*I* and Datasets S1 and S2).

Among our list of targets, we could identify sets of genes described to have a role in the developmental processes modified by *Ubx* in the haltere (Fig. 6 and Dataset S1). Ubx controls components of all major short- and long-range signaling path-

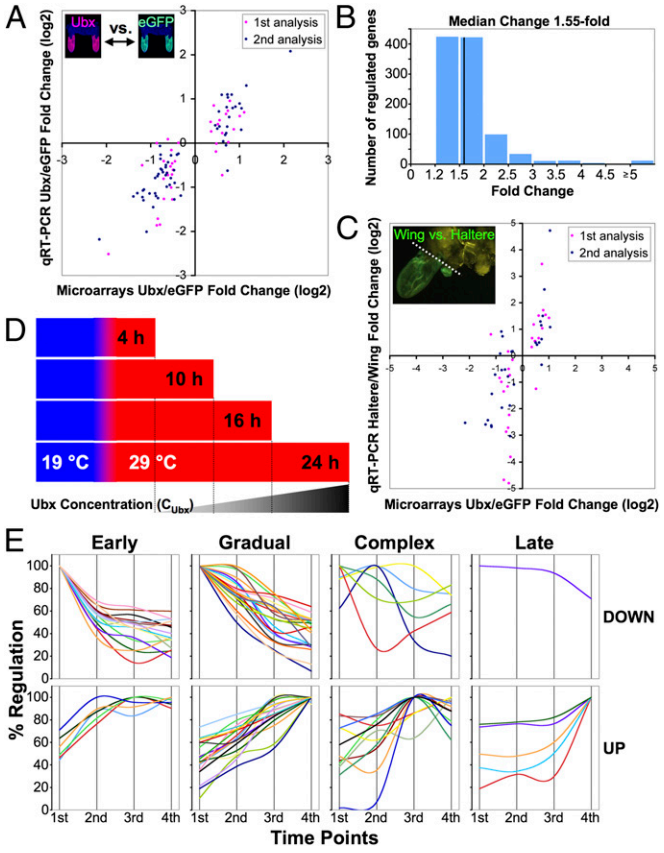

Fig. 5. Quantitative RT-PCR validation of Ubx targets. (*A*) The calculated fold change of target genes between *Ubx*- and *eGFP*-expressing wings collected 16 h ATS measured by qRT-PCR was plotted against the corresponding microarray measurement; each point represents measurements on a single gene at a single developmental stage (larval, prepupal, or pupal). Ubx targets identified only by the primary analysis are shown in magenta, and targets also identified by the second, more stringent analysis are in blue. (*B*) Frequency distribution of the fold changes (absolute values) for all genes recovered in the primary microarray analysis. (*C*) The microarray Ubx/eGFP fold change was plotted against the actual fold change between WT halteres and wings estimated by qRT-PCR. Each point represents measurements on a single gene at a single developmental stage (larval or prepupal; color-coded as in *A*). (*D*) Schematic representation of time series qRT-PCR assay. Animals grown at 19 °C (blue bars) were shifted to 29 °C (red bars). Wings were then collected 4, 10, 16, and 24 h ATS, times at which they are expressing 0%, 45%, 89%, and 101% of the Ubx protein levels (C_{Ubx}) observed in WT haltere capitellum, respectively. (*E*) Response profiles of target genes to Ubx. Each line shows the response of a single gene at a single developmental stage (larval, prepupal, or pupal) to rising concentrations of Ubx as determined by qRT-PCR. The first column shows genes with an early response at low Ubx concentrations, the second column shows genes with a gradual response to increasing Ubx concentrations, the third column shows genes with more complex responses to changing Ubx levels, and the fourth column shows genes with late responses at high Ubx levels. *Upper* panels show the responses of down-regulated genes, and *Lower* panels show the responses of up-regulated genes.

ways that are required for wing cell fate specification, cell proliferation and survival, and cell differentiation events. We found that Ubx controls the expression of many genes specifically involved in wing patterning and growth as well as general regulators of cellular growth and proliferation. Our analysis suggests that Ubx blocks vein/intervein differentiation in the haltere by directly regulating several genes required for establishment of longitudinal vein, cross-vein, and intervein territories in the wing. Ubx blocks the apposition of the dorsal and ventral wing surfaces in the haltere by targeting genes involved in basal cell adhesion. Ubx promotes major cytoskeletal reorganization in haltere cells relative to wing cells through direct regulation of genes encoding structural components of actin and microtubule filaments and accessory proteins controlling filament dynamics. Finally, Ubx suppresses development of bristle rows in the haltere by controlling many genes involved in wing margin formation.

Quantitative RT-PCR Confirms the Microarray Analysis. A large random fraction of the identified microarray targets with known biological function was validated by three different quantitative RT-PCR assays (Fig. 5 and Datasets S3, S4, and S5). The first test measured, in entirely independent samples, the fold change in transcript abundance between *Ubx*- and *eGFP*-expressing wings 16 h ATS. We collected 99 qRT-PCR measurements that were plotted against the microarray results (Fig. 5*A* and Dataset S3): the two sets of measurements were highly correlated (r = 0.89), with 89% of genes assayed (88 of 99) showing similar regulatory responses. The target genes identified by the more stringent analysis exhibited a higher correlation coefficient than those passing only the primary analysis (r = 0.92 vs. r = 0.80).

Most Target Genes in the Haltere Are Regulated by Ubx in a Subtle Fashion. The great majority of *Ubx* targets identified by our microarray approach exhibited relatively small changes in transcript abundance (median change = 1.55-fold, average change = 1.83-fold) (Fig. 5*B*). To further explore this finding, we carried out a second qRT-PCR analysis in which the microarray fold change for a given gene between *Ubx*- and *eGFP*-expressing wings was compared with its actual expression difference between WT halteres and wings (Fig. 5*C* and Dataset S4). This analysis showed that 84% (49 of 58) of the assayed genes were similarly up- or down-regulated in the two comparisons, but on average, the magnitude of the differential expression for these genes between halteres and wings was 4.3-fold (about 2.5 times higher than that observed in our temperature shift approach). We conclude that, in most cases, our approach revealed the partial rather than full response of target genes to Ubx.

Even considering this fact, the actual differences between WT halteres and wings observed by qRT-PCR still showed that the majority of assayed genes (69%; 34 of 49) were indeed regulated by Ubx in the haltere in a subtle fashion (less than fourfold difference), whereas 31% (15 of 49) were regulated by Ubx more strongly (4- to 28-fold difference) (Fig. 5*C*). This second assay also showed that the large majority of genes excluded by the second more stringent microarray analysis were indeed differentially expressed between WT wings and halteres (Fig. 5*C*) and thus, represent genuine Ubx targets.

Target Genes Exhibit Distinct Response Profiles to Ubx. Our experiments were designed to enrich for primary transcriptional responses to Ubx. To test how effectively we achieved this, we measured, by a third qRT-PCR approach, the expression levels of target genes at different time points ATS (Fig. 5*D*). Among putative targets assayed, 86% (86 of 100) exhibited the expected response to rising concentrations of Ubx protein as determined by the microarray analysis (Dataset S5). Of these 86 cases, the great majority (93%; 80 of 86) showed a clear response at early or intermediate time points (Fig. 5*E*), strongly suggesting that they are direct Ubx targets. Only a small fraction of genes (7%; 6 of 86) showed a much stronger response to Ubx after 24 than 16 h. These may be genes that respond only to high Ubx concentrations (like *knot*; compare Fig. 3 *L*, *L′*, and *L″* with its response curve in Fig. 5*E Upper*, fourth panel) or secondary targets that have crept through our screen. Overall, this time course analysis confirmed our original hypothesis that the 16-h time point chosen for the microarray analysis has been appropriate to primarily identify the direct targets of Ubx.

For most of the targets assayed, the early responses were amplified or refined after longer Ubx treatments, supporting the notion that high haltere-like Ubx levels and/or Ubx-dependent feed-forward loops are required to consolidate a particular reg-

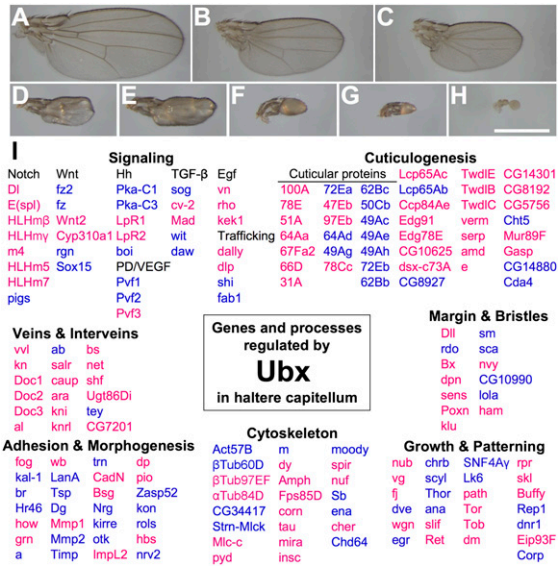

Fig. 6. Developmental processes and genes modulated by *Ubx* in halteres. (*A*–*G*) Homeotic transformation series of adult wings from the experimental *UAS-Ubx* line dissected from animals that were (*A*) developed exclusively at 19 °C or temperature shifted from 19 °C to 29 °C at (*B*) 36 h after puparium formation (APF), (*C*) 24 h APF, (*D*) 12 h APF, (*E*) at puparium formation, (*F*) 12 h before puparium formation (BPF), and (*G*) 36 h BPF. (*H*) Adult haltere. The observed homeotic transformations are stronger in earlier temperature shifts. Some processes, like cell-shape control and vein/intervein differentiation, are modulated by ectopic Ubx throughout the stages sampled, whereas others, like cell proliferation, marginal bristle formation, and adhesion of the dorsal and ventral wing surfaces, are sensitive to ectopic Ubx only during the larval and prepupal stages. All appendages are shown at the same scale. (Scale bar, 1 mm.) (*I*) Identified Ubx target genes with known biological functions have been grouped according to the process that they are likely carrying out in developing wings and halteres. To avoid multiple entries, components of signaling pathways and other pleiotropic genes have been assigned to a single group. Up-regulated genes are shown in blue, and down-regulated genes are in red.

ulatory output. This analysis also confirmed that we detected the partial and not full response for many genes with our microarray approach. Importantly, this assay enabled us to classify genes based on their response profiles to Ubx (Fig. 5*E*). It will be illuminating to study in the future how these classes relate to the architecture of the corresponding Ubx-responsive enhancers.

Discussion

This study represents a systematic effort to generate a comprehensive list of Hox targets over the course of an extended developmental process, namely the battery of genes targeted by *Ubx* during *Drosophila* haltere development. Our list of identified high-confidence Ubx targets in the haltere ranges from several hundreds to almost a thousand protein-coding genes. The wide range of genes targeted by Ubx matches the complexity of appendage morphogenesis and the numerous processes that have to be modulated to distinguish halteres from wings (Fig. 6). Our genome-wide study provides a firm demonstration of a view previously suggested by genetics that Ubx is a micromanager in the haltere: it modulates the wing program by interacting with the wing transcriptional network at multiple levels and over many developmental stages, controlling the expression of numerous target genes with a wide range of functions. However, there is also evidence for other modes of Hox gene action (4–8). For example, the role of Abdominal B in *Drosophila* posterior spiracles is more akin to that of a master control gene (30). In this context, the Hox protein activates a set of primary regulators at a specific time in development to initiate a novel organ. These few initial targets control terminal differentiation genes secondarily to orchestrate organogenesis. These distinct properties of Hox-controlled developmental programs will be governed by the architecture of the underlying transcriptional networks and the regulatory connections made by each Hox protein.

A striking aspect of our data is that the great majority of Ubx targets are stage-specific. The three time points that we have examined span the major hormonal events that drive metamorphosis. The late larval sample covers the role of *Ubx* during the final phase of patterning and growth of the imaginal disc epithelia up to the major release of the steroid hormone 20-hydroxyecdysone (20E) that triggers metamorphosis. During the prepupal period covered by the second sample, the epithelia respond to 20E by the morphogenetic movements of disc eversion and secretion of the pupal cuticle. The final sample is taken after the pupal molt, when the discs begin the final differentiation of the adult appendages. Our results show that the transcriptional responses to Ubx are quite different at these three time points. Further experiments are required to resolve to what extent the stage specificity of Ubx action reflects direct cross-talk between Ubx and hormonal cues through their combinatorial action on the Hox-targeted enhancers, to what extent it involves feed-forward loops of early targets jointly regulating later targets with Ubx, and whether it is also an indirect consequence of other changes in cell and chromatin states associated with developmental progression.

Our analysis has identified all genes previously showed as direct Ubx targets in the haltere pouch as well as a large fraction of suspected Ubx targets: *spalt*, *knot*, *blistered*, and *vestigial* (17, 19–21); *anachronism*, *CG16884*, *CG7201*, *CG8780*, and *CG11641* (27); the Egf receptor pathway components *vein*, *rhomboid*, and *kekkon-1* (22); *Cyp310a1*, *Delta*, *CG10990*, *CG5171*, and *E(spl)-C* genes (26); and *dally* and *dally-like* (14, 23–25). Most of the targets identified in our microarray analysis have not been studied thoroughly in the context of wing or haltere development, but some of these genes have been reported in microarray studies and genetic screens (31, 32).

Among the 872 target genes identified in our primary analysis, 90 targets (10%) have also been identified by either of two microarray comparisons of wing and haltere imaginal discs reported previously (26, 27). We conclude that many more genes identified in these previous genome-wide studies as differentially expressed between wing and haltere discs are not under direct Ubx control. We have noted that a few identified target genes show opposite responses to Ubx in our temperature shift experiments and WT halteres. For example, the gene *anachronism* has been classified as an up-regulated target in our primary analysis, although it has been shown to be repressed by Ubx in the haltere (27). Genes behaving similarly are filtered out from the list of Ubx targets in our secondary analysis but at the expense of removing many genuine Ubx targets.

Earlier studies have preferentially identified, as direct Ubx targets, genes that show striking expression differences between wings and halteres. Our approaches have enabled us to pick out not only these targets but also a much larger set of more subtly regulated transcriptional targets. Thus, it seems that *Ubx* controls complex morphogenetic and differentiation programs by modulating transcription of numerous target genes, switching a few of these genes on or off but regulating most targets in more subtle ways. Our findings generalize previous reports on the modulation of Dpp signaling by Ubx to control haltere size, which is brought about by the combined effects of subtle changes in the expression of several genes (14, 23–25).

We still need a detailed understanding of Hox-targeted enhancers to comprehend how Hox proteins achieve their biological activity. The few cases characterized to date suggest that Ubx regulation in the haltere capitellum occurs without cofactors through monomer TAAT core binding sites and that Ubx activity is highly context-dependent in the sense that the landscape of transcription factors and signaling molecules in a given cell at a given time guides specific regulatory effects (8, 19, 21, 27, 33). Complementing the present dataset with genome-wide chroma-

tin immunoprecipitation studies of Ubx occupancy in WT halteres and *Ubx*-misexpressing wings at different developmental stages should make it possible to locate the Ubx-responsive enhancers and will also help resolve longstanding debates concerning the molecular mechanisms underlying Hox binding selectivity and functional specificity.

Finally, ongoing comparative genomics with distantly related flies with larger genomes have the potential to systematically map enhancers in both *Drosophila* and these other species (34). Given that Hox-controlled gene circuits represent a favorable level for natural selection to drive the morphological and functional diversification of serial homologs, this cross-species comparison of the architecture of Ubx-regulated haltere networks will also help resolve how entire batteries of genes came under homeotic control to transform hind wings into halteres.

Materials and Methods

Animal Rearing and Sample Preparation. In all temperature shift experiments, crosses were kept at 19 °C and were shifted at the appropriate stage to 29 °C through a 1-h incubation at 25 °C to minimize heat-shock responses. Dissections were carried out in 1× PBS equilibrated to the same temperature as the flies to be processed. More details are in *SI Materials and Methods*.

Affymetrix *Drosophila* Genome 2.0 Array Data Analysis. For the identification of differentially expressed genes, we processed expression data of the two compared replicate groups—linked with double arrows in Fig. 4 *A* and *C*—by the robust multiarray average (RMA) method, filtered out the Affymetrix quality control probe sets and genes that were not expressed above 5 $\log_2$ units in at least two of eight samples, and identified the up- and down-regulated genes using the rank products nonparametric method. For each Affymetrix probe set, FDR was computed from 1,000 permutations; a significance cutoff of 5% was used in the primary statistical analysis (Fig. 4*A*), and a less strict cutoff of 10% was used in the first part of the secondary analysis (Fig. 4*C*). GO analyses were carried out with Genespring GX 11.0, which calculates the uncorrected and Benjamini–Yekutieli adjusted over-representation probabilities for each GO term. Dataset S2 shows GO terms enriched in the entire list and in stage-specific sets of Ubx targets with uncorrected $P < 0.01$. More information is in *SI Materials and Methods*.

We have noted, in our primary microarray analysis, the up-regulation of genes mediating stress responses, particularly in the larval stage. Presumably, these responses represent side effects of the temperature shift protocol rather than targets of Ubx. We have also discovered a few genes known to play roles in tracheal and muscle development. These genes may represent artifacts of the dissected material, because the developing dorsal thoracic air sac and flight muscles are closely associated with the developing wing epithelium.

Immunofluorescence and in Situ Hybridization. We used the FP3.38 antibody against Ubx at 1:10, FITC-phalloidin at 1:50 (Sigma), AlexaFluor-conjugated secondary antibodies at 1:500 (Invitrogen), sheep anti–digoxigenin-alkaline phosphatase antibody at 1:3,000 (Roche), and TO-PRO-3 iodide at 1 μM (Invitrogen). More details are in *SI Materials and Methods*.

Quantitative RT-PCR. Relative quantification of transcript levels was performed on a 480 LightCycler (Roche) using SYBR Green I assays. The expression levels of each target gene were normalized against the expression levels of the *RpL21* reference gene, which was one of the least changing genes across all 48 arrays. All primer sequences and reaction conditions are available on request.

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